Partial Characterization of the Intra- and Extracellular Acid Phosphatase of an Alga, *Ochromonas danica*

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SUMMARY

Intra- and extracellular acid phosphatases were purified 88- and 65-fold respectively from photoheterotrophic *Ochromonas danica* Pringsheim. The purified enzymes differed in heat inactivation, substrate specificity, and inhibition by several divalent cations and NaF. Intracellular enzyme lost only 30 % of its activity by heating at 60 °C for 200 min whereas the extracellular enzyme lost 80 %. Both enzymes were active over a broad pH range from 2.2 to 5.2 and had an optimum pH of 4.8. Both had broad substrate specificity and differed in their relative ability to hydrolyse β -glycerophosphate, phenolphthalein diphosphate, glucose-1-phosphate, fructose-1,6-diphosphate, ADP and ATP. Both were inhibited by Co^{2+} , Zn^{2+} , Hg^{2+} , Fe^{3+} , arsenate, tartrate and fluoroacetate but differed in their inhibition by Cu^{2+} , Hg^{2+} and NaF. Intracellular acid phosphatase was more susceptible to inhibition by Hg^{2+} and NaF, while extracellular acid phosphatase was more susceptible to inhibition by Cu^{2+} . *p*-Chloromercuribenzoate and urea had no effect on either enzyme's activity. EDTA stimulated the activity of both enzymes. The *K_m* for the intra- and the extracellular enzymes was 0.5 and 0.33 mM respectively with *p*-nitrophenyl phosphate as the substrate.

INTRODUCTION

Exoenzymes (extracellular enzymes) are commonly found in bacteria (Pollock, 1962; Lampen, 1965) and protozoa (Müller, 1967) but have not been demonstrated critically in algae or phytoflagellates although several claims have been made (see reviews by Fogg, 1962; Aaronson, 1973). Recently we described the existence of several exoenzymes in the culture filtrate of the phytoflagellate *Ochromonas danica* (Aaronson, 1971; Aaronson, 1973). In this paper we present biochemical evidence for the existence of distinct intra- and extracellular acid phosphatases in *O. danica*.

METHODS

Organism and growth condition. *Ochromonas danica* L933/2 Pringsheim was maintained in a chemically defined medium (Aaronson & Baker, 1959) in 5 ml volumes in screw-cap test tubes in a refrigerated incubator at 25 °C with 125 to 150 ft-candle of white fluorescent light. Experimental cultures were grown similarly in 500 ml volumes in 1 l, screw-cap Erlenmeyer flasks. Flasks were inoculated with 20000 organisms/ml from a 48 to 72 h culture and incubated for 5 days (exponential-phase organisms).

Harvesting and preparation of extracts. All centrifugation procedures were carried out at 4 °C. Exponential-phase organisms were harvested by centrifugation at 4340 *g* for 20 min in a Sorval RC-2B refrigerated centrifuge. The organisms were resuspended in 40 ml cold distilled water and homogenized with glass beads at 20000 rev./min for 30 s in a Sorval

Omnimixer. The homogenate was kept cold in an ice bath during homogenization. Glass beads and debris were removed by centrifugation at 4340 *g* for 20 min and the supernatant, after appropriate dilutions, was used immediately for the enzyme assay of intracellular acid phosphatase or frozen (-10 to -15°C) and stored for later use. Supernatant obtained after harvesting the exponential-phase organisms was used for the assay of extracellular acid phosphatase secreted into the growth medium.

Enzyme assays and analytical procedures. Acid phosphatase activity was routinely assayed in duplicate by incubating the enzyme for 10 to 15 min at 37°C with *p*-nitrophenyl phosphate as substrate. The assay mixture (1.0 ml total volume) contained 0.5 ml 15 mM-*p*-nitrophenyl phosphate in acetate buffer, 0.3 ml 0.1 M-acetate buffer pH 3.6, 0.2 ml homogenate which contained 20 to 30 μg total protein or 0.2 ml supernatant for assay of extracellular acid phosphatase. The reaction was initiated by the addition of substrate and was terminated by the addition of 5 ml 0.1 M-NaOH. The *p*-nitrophenol released by the enzyme was measured at 410 nm in a Hitachi-Perkin Elmer Model 139 spectrophotometer by the method of Bessey, Lowry & Brock (1946). The rate of reaction was linear with respect to time and enzyme concentration under these conditions.

Enzyme activity towards a number of other phosphate esters was determined by the liberation of inorganic phosphate. Assay mixtures (1.0 ml total volume) contained 0.5 ml substrate solution in buffer, 0.3 ml 0.1 M-acetate buffer pH 3.6, and 0.2 ml enzyme solution. After incubation for 10 to 15 min the reaction was stopped by the addition of 1.0 ml 10 % trichloroacetic acid. The liberated inorganic phosphate was determined by the method of Fiske & SubbaRow (1925) or by the method of Chen, Toribara & Warner (1956) as modified by Ames & Dubin (1960). Enzyme units are expressed as μmol *p*-nitrophenol or inorganic phosphate liberated per min; the specific activity was defined as the enzyme units per ml culture or per mg protein.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin (Sigma) as a standard. Buffers were prepared according to the procedures of Gomori (1955). All compounds used were of the highest purity available commercially and were used without further purification.

Purification methods. All preparative procedures were carried out at 0 to 5°C unless otherwise indicated.

Ammonium sulphate fractionation. Solid $(\text{NH}_4)_2\text{SO}_4$ (243 mg) was added to each ml of the crude extract. After 1 h of stirring, the proteins that precipitated were removed by centrifugation for 20 min at 25000 *g*. The supernatant was adjusted to 90 % saturation by the addition of 375 mg $(\text{NH}_4)_2\text{SO}_4$ to each ml. The protein precipitated by this procedure was dissolved in 0.05 M-acetate buffer (pH 4.8) so that the final protein concentration was 19.5 mg/ml. After dialysis against the same buffer the solution was centrifuged. All the activity was in the supernatant fraction.

In the case of extracellular acid phosphatase, 313 mg of solid $(\text{NH}_4)_2\text{SO}_4$ was added to each ml of a 5-day-old cell-free culture medium to give 50 % saturation. After 1 h, precipitated protein was removed by centrifugation and the supernatant was adjusted to 90 % saturation by adding 302 mg $(\text{NH}_4)_2\text{SO}_4$ to each ml of supernatant. After 1 h of stirring precipitates were recovered by centrifugation. The pellet was dissolved in 0.05 M-acetate buffer (pH 4.8) to give a final protein concentration of 8.7 mg/ml.

Chromatography on Sephadex G-100. Sephadex G-100 (30 g) was allowed to swell in water and washed according to the manufacturer's directions. The slurry was washed twice with 1 l 0.05 M-acetate buffer (pH 4.8) and allowed to equilibrate in the same buffer overnight. The enzyme solution from the previous step (6 ml) was applied to a column

Table 1. *Summary of purification of intracellular acid phosphatase*

For experimental details see the text.

Step no.	Fractions	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity	Recovery (%)	Purification
1	Crude extract	500	223.7	17.0	13.16	100.0	1
2	40–90 % saturation (NH ₄) ₂ SO ₄	7.0	1114.0	19.5	57.13	69.7	4.34
3	Pooled fractions from Sephadex G-100	48.0	101.67	0.22	462.14	43.6	35.12
4	Second run on Sephadex G-100						
	11	4.5	316.9	0.368	861.1	12.7	65.4
	12	4.5	329.7	0.282	1169.1	13.2	88.8
	13	4.5	234.6	0.220	1066.4	9.4	81.03
	14	4.5	104.9	0.112	936.6	4.2	71.17

(2.5 × 82.0 cm) with the acetate buffer at 4 °C and eluted with the same buffer at a flow rate of about 8 ml/h. Fractions of 4.5 ml were collected and the enzyme was eluted between fractions 20 and 50. The most active fractions (25 to 36) were pooled and solid (NH₄)₂SO₄ added. The precipitate, with 40 to 90 % saturation, was dissolved in 6 ml buffer and applied to a column (2.5 × 35 cm) of Sephadex G-100 equilibrated against acetate buffer, pH 4.8. The column was eluted at 4 °C with the same buffer and 4.5 ml fractions were collected. The void volume was about 55 ml.

For purification of extracellular acid phosphatase, the enzyme solution from the previous step (8 ml) was applied to a column of Sephadex G-100. Enzyme was eluted between fractions 21 and 49. The most active fractions (41 to 44) were pooled. Solid (NH₄)₂SO₄ was added to the pooled fractions. The precipitate with 50 to 90 % saturation was dissolved in 6 ml acetate buffer (pH 4.8) and applied to a column of Sephadex G-100.

RESULTS

Purification

The results of a typical purification of the intracellular acid phosphatase are shown in Table 1. The highest final purification varied from 65- to 88-fold purification in different fractions. The specific activity of the purified enzyme varied between 861 and 1169 units/mg protein. The highest final purification of the extracellular acid phosphatase (Table 2) varied from 44- to 66-fold purification in different fractions. The specific activity of the purified enzyme varied between 970 and 1427 units/mg protein. The elution profile for intra- and extracellular acid phosphatase is shown in Fig. 1.

Properties of the intra- and extracellular acid phosphatase

Because of the very small quantities of purified enzyme obtained, only the properties of critical importance, i.e. differences in the heat stability, substrate specificity, inhibition and kinetics, were confirmed by using the purified intra- and extracellular acid phosphatase preparations. Therefore, unless otherwise stated, the results expressed here represent the properties of the intra- and extracellular acid phosphatase when using homogenate and culture medium as the enzyme source.

Temperature stability. Both purified and crude intra- and extracellular acid phosphatase were stable for at least two months when stored at –10 to –15 °C in 0.05 M-acetate buffer,

Table 2. *Summary of purification of extracellular acid phosphatase*

For experimental details see the text.

Step no.	Fractions	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity	Recovery (%)	Purification
1	Supernatant	12480	3.9	0.180	21.66	100.0	1
2	50-90 % saturation $(\text{NH}_4)_2\text{SO}_4$	62	705.4	8.75	80.62	89.8	3.72
3	Pooled fractions from Sephadex G-100 column	16	161.9	0.348	465.23	5.3	21.48
4	Second run on Sephadex G-100						
	14	4.5	24.25	0.025	970.0	0.2	44.78
	15	4.5	57.1	0.04	1427.5	0.5	65.9
	16	4.5	41.4	0.035	1182.8	0.3	54.6
	17	4.5	27.1	0.025	1084.0	0.25	50.05

Fig. 1. Elution of purified intracellular (b) and extracellular (a) acid phosphatase from Sephadex G-100. Details of the procedures used are given in the text. ●, Enzyme activity; ○, protein.

Fig. 2. Thermal denaturation of the phosphatase activity. The enzyme preparations (cell homogenate and cell-free supernatant) containing about 30 μ g total protein, were heated at 60 °C in 0.1 M-acetate buffer (pH 3.6) for the times indicated. The heating was terminated by transferring a sample into an icebath, and the remaining activity was assayed at 37 °C as described under the standard assay conditions. ●, ○, Intra- and extracellular acid phosphatase activity, respectively.

pH 4.8. Freezing and thawing had no effect on the enzymic activity. A 15 to 20 % loss in activity of intra- and extracellular enzyme was observed when enzymes were stored at 0 to 5 °C for two months. The effect of heat treatment of the homogenate and culture supernatant is illustrated in Fig. 2. Heating the enzyme solution for 200 min at 60 °C caused about 30 % inactivation of the intracellular acid phosphatase activity; the extracellular enzyme lost about 80 % of its activity. Complete loss of intra- and extracellular acid phosphatase activity was observed when enzyme solution was heated at 80 °C or above for 20 min. The homogenate was heated for 1 h in a boiling water bath, centrifuged, and the supernatant added to the extracellular enzyme solution before thermal denaturation. No protection of the extracellular enzyme by the heated cell homogenate fraction was observed. Despite their differences in thermal stability, intra- and extracellular acid phosphatases had the same temperature optima. A 2- to 2.5-fold increase in the specific activity of both enzymes was observed at 55 °C as compared with the specific activity at 37 °C when using *p*-nitrophenyl phosphate as substrate. Above 60 °C the activity of intra- and extracellular enzymes declined sharply.

pH optimum. Both intra- and extracellular acid phosphatases had a broad pH range from 2.2 to 5.2 with an optimum at 4.8 (Fig. 3). At higher pH, phosphatase activity fell very sharply. Only about a 5.0 % difference in activity was observed between pH 2.2 and 5.2,

Fig. 3. pH-activity curve for partially purified acid phosphatase. Intracellular (specific activity 1066) and extracellular (specific activity 1084) enzyme preparations were assayed directly at various pH values in the following buffers at 0.1 M final concentrations: 2.6 to 3.6, glycine-HCl; 3.6 to 5.6, acetate buffer; 5.2 to 7.0, tris-maleate. ●, ○, Intra- and extracellular acid phosphatase activity, respectively.

Table 3. *Substrate specificity of intra- and extracellular acid phosphatases of Ochromonas danica*

All substrates were assayed at 37 °C in 0.1 M-acetate buffer at pH 3.6. The substrate concentration was 7.5 mM. The specific activities of intra- and extracellular phosphatase with *p*-nitrophenyl phosphate as substrate were 12.0 and 4.9 μ mol inorganic phosphate liberated/min/ml culture, respectively. The rate of *p*-nitrophenyl phosphate hydrolysis for each enzyme was considered to be 100 % and the other values are indicated as percentages of this value.

Substrate	Relative rate of hydrolysis	
	Intracellular	Extracellular
<i>p</i> -Nitrophenyl phosphate	100.0	100.0
β -Glycerophosphate	106.0	126.7
Phenolphthalein diphosphate	89.0	44.8
Glucose 6-phosphate	97.5	94.0
Glucose 1-phosphate	69.5	53.6
Fructose 1,6-diphosphate	50.5	67.7
Adenosine monophosphate	114.0	114.4
Adenosine diphosphate	48.5	20.0
Adenosine triphosphate	9.1	0.0

Fig. 4. Effect of (a) NaF, (b) HgCl_2 , and (c) CuCl_2 , on the intra- and extracellular acid phosphatase activity. Enzyme was preincubated at 37°C for 20 min with varying concentrations of cations before the addition of *p*-nitrophenyl phosphate as substrate. ●, ○, Intra- and extracellular acid phosphatase activity, respectively.

and all subsequent assays were done at pH 3.6. Similar broad-range pH activity was observed with unpurified homogenate and medium.

Substrate specificity. Both enzymes differed in their ability to hydrolyse β -glycerophosphate, phenolphthalein diphosphate, glucose 1-phosphate, fructose 1,6-diphosphate, ADP and ATP (Table 3). Both enzymes were unable to hydrolyse DNA or lecithin.

Effect of inhibitors. Cations (at 5 mM) were incubated at 37°C for 20 min before addition of *p*-nitrophenyl phosphate as substrate. The presence of K^+ , Mg^{2+} , Ca^{2+} or Mn^{2+} had no significant effect on either enzyme, whereas Co^{2+} , Zn^{2+} , Hg^{2+} and Fe^{3+} were equally inhibitory to both enzymes. Extracellular acid phosphatase was more susceptible to inhibition by Cu^{2+} (Fig. 4c), while Hg^{2+} was more inhibitory to the intracellular acid phosphatase (Fig. 4b).

Table 4. *Heat stability of purified intra- and extracellular acid phosphatases*

We used 4.0 μ g protein/assay system. Purified enzyme in 0.1 M-acetate buffer (pH 3.6) was heated for different time intervals at 60 °C. Enzyme activity was assayed at pH 3.6 using *p*-nitrophenyl phosphate as substrate. Results are expressed as relative activity, taking the activity of unheated enzyme as 100 %. Variations express differences in the relative activity of purified enzymes as compared with the activity of crude cell homogenate or supernatant.

Time of inactivation (min)	Relative activity	
	Intracellular	Extracellular
0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
40	90.6 $\pm$ 1.1	65.8 $\pm$ 2.9
120	78.35 $\pm$ 0.2	29.0 $\pm$ 3.9
240	72.15 $\pm$ 0.7	20.25 $\pm$ 3.5

Table 5. *Effect of different inhibitors on purified intra- and extracellular acid phosphatases*

We used 4.0 μ g protein/assay system. Enzyme solution was preincubated with varying concentrations of inhibitors for 20 min at 37 °C prior to the addition of *p*-nitrophenyl phosphate (7.5 mM) as substrate. Assay was done using 0.1 M-acetate buffer at pH 3.6. Results are expressed as percentage of relative activity, taking activity with *p*-nitrophenyl phosphate, without addition of an inhibitor, as 100 %. Other details are as in Table 4.

Additions	Concentration (mM)	Relative activity	
		Intracellular	Extracellular
None	—	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
CuCl ₂	1.0	100.0 $\pm$ 0.0	91.25 $\pm$ 2.55
	5.0	97.25 $\pm$ 0.45	67.5 $\pm$ 4.5
	7.0	90.35 $\pm$ 14.65	20.35 $\pm$ 2.85
HgCl ₂	1.0	63.5 $\pm$ 17.0	81.0 $\pm$ 2.35
	3.0	4.5 $\pm$ 7.0	60.1 $\pm$ 1.45
NaF	0.01	43.4 $\pm$ 8.6	81.0 $\pm$ 1.7
	0.02	19.85 $\pm$ 5.25	64.8 $\pm$ 2.4

Arsenate, tartrate, fluoroacetate, fluoride and iodoacetic acid were found to be potent inhibitors for both enzymes, unlike azide, cystein-HCl, iodoacetamide, *p*-chloromercuribenzoic acid, citrate, urea or EDTA. Fluoride inhibited the intracellular activity more (Fig. 4*a*). Phosphatase activity was stimulated by chelating agents including EDTA, suggesting that metal ions were not essential for the enzyme activity of both intra- and extracellular acid phosphatase.

Effects of purification. The differences between intra- and extracellular acid phosphatase reported above might be due to an artefact of the crude enzyme preparations. Hence, some of the properties were investigated with the partially purified preparations.

After heating the enzyme at 60 °C for 240 min, about 30 % of intracellular enzyme activity was lost as compared to almost 80 % inactivation of the extracellular acid phosphatase (Table 4). A purified enzyme preparation was preincubated with different concentrations of Cu²⁺, Hg²⁺ and NaF for 20 min at 37 °C before the addition of *p*-nitrophenyl phosphate to study differences in inhibition (Table 5). The minimum and maximum variation observed between the properties of purified and crude enzymes were in the range 0.45 to 8.6 %, the only exception being the inhibition with CuCl₂ (7 mM) and HgCl₂ (1 mM) when the variation in the activities was 14 to 17 %. The intracellular enzyme was appreciably more active with phenolphthalein diphosphate, glucose-1-phosphate or ADP, while the extracellular enzyme was more active with fructose-1,6-diphosphate. The results presented

Table 6. *Effect of mixing the intra- and extracellular enzyme preparations on the differential inhibition of intracellular, extracellular and mixed enzyme preparations*

Two ml purified intracellular enzyme preparation (20 µg protein/ml) was mixed with 2 ml of culture supernatant as extracellular enzyme. Four ml each of purified intracellular enzyme, culture supernatant and equally mixed (intra- and extracellular) enzyme preparation were incubated at 25 °C in a refrigerated incubator with 125 to 150 ft-candle fluorescent light. Enzyme samples were removed at 0, 4, 8 and 24 h, preincubated with 5 mM-CuCl₂, 1 mM-HgCl₂, and 10 µmol NaF individually for 20 min at 37 °C, and assayed for the remaining activity with *p*-nitrophenyl phosphate as substrate. Activity is expressed as absorbance at 410 nm.

Time of incubation at 25 °C (h)	I Intracellular activity (absorbance)				II Intra- and extracellular activity (absorbance)				III Extracellular activity (absorbance)			
	Control	CuCl ₂	HgCl ₂	NaF	Control	CuCl ₂	HgCl ₂	NaF	Control	CuCl ₂	HgCl ₂	NaF
0	0.350	0.345	0.150	0.130	0.300	0.250	0.195	0.170	0.280	0.195	0.240	0.220
4	0.350	0.340	0.140	0.135	0.300	0.255	0.200	0.180	0.285	0.200	0.235	0.215
8	0.340	0.340	0.145	0.130	0.295	0.250	0.190	0.170	0.275	0.205	0.230	0.210
24	0.320	0.320	0.135	0.120	0.280	0.235	0.180	0.170	0.260	0.200	0.210	0.205

above, and in Tables 3, 4 and 5, show that differences between the substrate specificity and inhibition properties of intra- and extracellular acid phosphatases were not due to an artefact of the crude enzyme preparations.

Possibility of conversion of intra- to extracellular enzyme. Purified intracellular acid phosphatase preparation (I) was mixed with an equal volume of crude extracellular enzyme (II), and the mixture incubated at 25 °C for 24 h under conditions similar to that for the growth of the culture. As a control, purified intracellular preparation (I) and cell-free supernatant (III) were incubated separately under similar conditions. At different time intervals (0, 4, 8 and 24 h) activity was assayed, without and with inhibitors, with *p*-nitrophenyl phosphate as substrate. Within 24 h incubation (Table 6), there was no difference in the inhibitory pattern of the various enzyme preparations. Inhibition of the mixed enzyme activity was about half-way between the inhibition of intra- and extracellular acid phosphatases separately. From these results we infer that the differences between the intra- and extracellular acid phosphatases are not due to the conversion of one enzyme into the other. Similar results were obtained when purified extracellular enzyme was incubated under the same conditions with an equal quantity of crude intracellular acid phosphatase.

Kinetic studies. When the activities of purified enzymes were measured at various concentrations of *p*-nitrophenyl phosphate as substrate, a double-reciprocal plot (Lineweaver & Burk, 1934) gave a straight line (Fig. 5). The K_m with intra- and extracellular acid phosphatases was 0.5 and 0.33 mM respectively.

DISCUSSION

The properties of the intra- and extracellular acid phosphatases of *Ochromonas danica* described in this paper demonstrate that this alga secretes an enzyme into its environment which is distinctly different from the enzyme inside the cell. Different intra- and extracellular acid phosphatases in another micro-organism, *Aspergillus oryzae*, have also been reported (Sakurai & Shiota, 1970).

Unlike the enzyme from the fungus *Fusarium moniliforme* (Yoshida & Tamiya, 1971), which was stable up to 40 °C at pH 5.3, the intra- and extracellular acid phosphatases of *Ochromonas danica* were completely stable at 55 °C, pH 3.6. Both enzymes of *O. danica* differed from the induced acid phosphatase of the phytoflagellate *Euglena gracilis* (Bennun

Fig. 5. Rate versus substrate concentration for intra- and extracellular acid phosphatases. The reaction mixture contained in a total volume of 1.0 ml: 3.3 or 1.2 μ g partially purified intra- and extracellular acid phosphatases, 0.3 ml 0.1 M-acetate buffer (pH 3.6), and different concentrations of *p*-nitrophenyl phosphate in buffer as substrate. Incubation was for 10 min at 37 °C. Substrate concentration is expressed in mM and initial velocity is expressed as mm-*p*-nitrophenol liberated/min/mg protein. ●, ○, Intra- and extracellular acid phosphatase, respectively.

& Blum, 1966), which lost almost all activity within 17 min at 60 °C. Acid phosphatase of the fungus *Aspergillus nidulans* (Harsanyi & Dorn, 1972) lost about 50 % activity when heated at 50 °C. The enzymes of *O. danica* were active at a wide pH range from 2.2 to 5.2 and the optimum pH for both enzymes was about 4.8. Similar optima for acid phosphatase have been reported for *E. gracilis* (Blum, 1965), the ciliate *Tetrahymena pyriformis* (Müller, Baudhuin & De Duve, 1966; Lazarus & Scherbaum, 1967), *Acanthamoeba* sp. (Müller, 1969), the green alga *Chlorella pyrenoidosa* (Knutsen, 1968), the fungus *F. moniliforme* (Yoshida & Tamiya, 1971), and human erythrocyte acid phosphatase (Fenton & Richardson, 1971) and bone tissue acid phosphatase (Vaes & Jacques, 1965). An optimum pH of 3.2 has been reported for the acid phosphatase of the amoeba *Mayorella palestinensis* (Lasman, 1967) while that of *Escherichia coli* has an optimum pH of 2.5 (Hafkenscheid, 1968).

The insensitivity of intra- and extracellular acid phosphatase of *Ochromonas danica* to metal-binding agents like EDTA implies that these enzymes are not metalloenzymes like the alkaline phosphatase of *Escherichia coli* (Plocke, Levinthal & Vallee, 1962). Similar results have been reported for the induced acid phosphatases of *Euglena* (Bennun & Blum, 1966), *Tetrahymena pyriformis* (Lazarus & Scherbaum, 1967), *Aspergillus nidulans* (Harsanyi & Dorn, 1972) and hog spleen acid phosphatase (Chersi, Bernardi & Bernardi, 1966). Like the acid phosphatase of *Staphylococcus aureus* (Malveaux & San Clemente, 1969) and bovine-liver acid phosphatase (Heinrikson, 1969), both *O. danica* acid phosphatases were activated by EDTA. Fluoride, tartrate and arsenate inhibit many acid phosphatases (Torriani, 1960) as well as the intra- and extracellular acid phosphatases of *O. danica*. Complete inhibition of acid phosphatase by fluoride is also reported in *Mayorella palestinensis* (Lasman, 1967), the amoeba *Chaos chaos* (Holter & Lowy, 1959) and *Salmonella*

typhimurium (Carillo-Castaneda & Ortega, 1967). Blum (1965) observed that fluoride inhibited the induced and constitutive acid phosphatase activity of *Euglena*. Fluoride at μmol levels inhibited *O. danica* intra- and extracellular acid phosphatases, the intracellular enzyme being more susceptible to inhibition than the extracellular acid phosphatase. Tartrate inhibited the intra- and extracellular acid phosphatases of *O. danica* and the acid phosphatase of *Neurospora* (Nyc, 1967) and *A. nidulans* (Harsanyi & Dorn, 1972) but had no effect on the acid phosphatases of *Saccharomyces cerevisiae* (Kilsheimer & Axelrod, 1957, 1958) or *S. aureus* (Malveaux & San Clemente, 1969).

The relative specificity of intra- and extracellular acid phosphatases of *Ochromonas danica* for several substrates indicated little about the natural substrate and role of the enzymes *in vivo*. The substrate specificity of the intra- and extracellular acid phosphatase was very broad; this is also true of the induced enzyme of *Euglena* (Bennun & Blum, 1966). However, the enzymes of *Euglena gracilis* could hydrolyse pyrophosphate much more than *p*-nitrophenyl phosphate. The acid phosphatase of *Fusarium moniliforme* (Yoshida & Tamiya, 1971) and *Salmonella typhimurium* (Carillo-Castaneda & Ortega, 1967) could also hydrolyse pyrophosphate, ATP and ADP more actively than *p*-nitrophenyl phosphate. Hydrolysis of pyrophosphate is also reported for the acid phosphatase from tobacco cells (Ueki & Sato, 1971) and sweet potato (Uehara, Fujimoto & Taniguchi, 1971). In contrast, both intra- and extracellular acid phosphatases of *O. danica* were unable to hydrolyse pyrophosphate, and ATP was hydrolysed about 10 % with only the intracellular enzyme. Both phosphatases of *O. danica* are more active with the hexose phosphates, AMP and ADP, as substrates than are the acid phosphatase of *F. moniliforme* (Yoshida & Tamiya, 1971) and *Escherichia coli* (Hafkenschied, 1968).

Despite its clear distinction from the intracellular acid phosphatase and similarity to acid phosphatases in other micro-organisms, the role of the extracellular acid phosphatase in *Ochromonas danica* remains to be determined. To our knowledge this is the first clear demonstration of an extracellular enzyme in an alga (phytoflagellate).

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